

1 **Embryo metabolism: what does it really mean?**

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21 **Abstract**

22 The study of early embryo metabolism has fascinated researchers in the field for nearly a
23 century. Herein, we give a brief account of the general features of embryo metabolism and
24 some consideration of the research performed to reach such conclusions. It is becoming
25 increasingly obvious that metabolism informs many fate decisions and outcomes beyond
26 ATP generation, such as DNA methylation, Reactive Oxygen Species generation and cell
27 signaling. We discuss the reasons for studying metabolism in the face of our current
28 knowledge of the effect that the culture environment on the developing embryo and the
29 downstream effects that can cause. The study of *in vitro* embryo metabolism can also give
30 us insight into developmental perturbations *in vivo*. The strengths and limitations of the
31 methods we use to study metabolism are reviewed with reference to species-specific
32 fundamental biology and plasticity and we discuss what the future holds for metabolic
33 studies and the unanswered questions that remain.

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35

36 **Introduction**

37 The study of mammalian early embryo metabolism has a rich history (Leese, 2012).
38 Whilst work in the period of the 1940s-1960s focused on the effect of adding energy
39 substrates to embryos in culture, real progress in understanding embryo metabolism was
40 made in the 1970s by the likes of Biggers and Stern (1973), Brinster (1973) and Gwatkin
41 and Haidri (1974) who examined the fate of radiolabeled compounds added to the
42 medium. From experiments such as these, a picture of early embryo metabolism began to
43 emerge. Like so much of our knowledge of early mammalian embryo development, the
44 first data came from the classical laboratory model species; mouse and rabbit, as well as
45 the hamster. Interest grew, and embryo metabolism was soon examined in the large
46 domestic animals; pigs, cattle, sheep and, to a lesser extent, the horse, dog and cat.
47 Underpinning research were studies on early human development with the aim of clinical
48 translation for the treatment of infertility; a feat first achieved in 1978 by Steptoe and
49 Edwards. Alongside this feat was the development of assisted conception techniques for
50 use in farm animals. It is not the intention of this article to re-describe the history of the
51 research that lead to successful embryo culture or the contribution that studies on
52 metabolism. For expert insight, the reader is encouraged to read (Leese, 2012;
53 Chronopoulou and Harper, 2014).

54

55 **Embryo metabolism: what do we know?**

56 The description of carbohydrate metabolism during preimplantation development is largely
57 accepted and will be familiar to anyone who has an interest in the early embryo. In almost
58 all species studied, the cleavage stage embryo, from fertilisation through to formation of
59 the morula, is relatively metabolically quiescent. Oxygen consumption at this time remains
60 comparatively low, and the dominant substrate depleted from the culture environment is

pyruvate. Pyruvate is consumed at an almost steady rate during cleavage, with a proportion of the carbon (depending on the species) appearing in the medium as lactate with the generation of metabolic energy. The source of the pyruvate involved in such reactions is generally either glycolytic conversion of glucose or that taken up directly from the external environment. Pyruvate may also enter the Tricarboxylic Acid (TCA; Krebs) cycle, where it can be oxidised completely generating electron donors for the electron transport chain which occurs in the matrix of mitochondria and relies on oxygen acting as the terminal electron acceptor. For this reason, oxygen consumption provides a good marker of overall oxidative metabolic activity (for review, see Smith and Sturmey, 2013).

As the cleavage stage embryo progresses to a blastocyst, there is a sharp and characteristic rise in the amount of glucose consumed in all species studied, and a concomitant rise in lactate release into the medium. Coincident with this is a fall in pyruvate consumption. This general pattern of “blastocyst glycolysis” appears to be conserved across all species studied. There are a range of explanations for this, however, as glycolysis is a comparatively inefficient means of generating ATP therefore energy production is unlikely to be the prime reason. Moreover, as the blastocyst forms, oxygen consumption also rises (Fridhandler *et al.*, 1957, Houghton *et al.*, 1996, Sturmey and Leese, 2003, Thompson *et al.*, 1996, Trimarchi *et al.* 2000) further supporting the notion that glycolytic production of lactate is of minor consequence in contributing ATP for the blastocyst. It is much more likely that glycolysis rises to meet the need for carbon for biosynthetic processes. A description of glycolysis in the early embryo can be found in Smith and Sturmey (2013). This general picture of embryo metabolism was summarized with great prescience by Brinster in 1973; in the intervening years many laboratories

85 across the world have generated evidence to support such a description, illustrating the
86 robustness with which these findings can be considered.

87

88 While early work focused on carbohydrate metabolism, it is now clear that the metabolism
89 of amino acids, lipids and vitamins such as folate all also act in an interdependent manner
90 to produce a viable embryo. Amino acids are crucial components of the culture
91 environment *in vitro* (reviewed by Sturmey *et al.*, 2010). Their addition to simple culture
92 medium either singly (Rieger *et al.*, 1992) or in combinations (Chatot *et al.*, 1989, Gardner
93 and Lane, 1993) permitted mouse embryos to be cultured past the so-called 2-cell block
94 (Chatot *et al.*, 1989) and their widespread inclusion lead to improved blastocyst rates in
95 almost every species studied. The addition of amino acids has had such a positive effect
96 on the efficacy of *in vitro* embryo culture, that their inclusion is often described as having a
97 primary role in the formulation of “next generation medium” (Leese, 2012). The precise
98 mechanism for the positive effect of amino acid provision is still to be defined, however it is
99 well established that addition of amino acids to *in vitro* medium can alleviate culture
100 associated stress in flushed murine embryos (Lane and Gardner, 1998). The contribution
101 that amino acid metabolism makes to ATP production remains unclear, however the
102 turnover of amino acids (that is, the sum of their depletion or accumulation into the culture
103 droplet) has been linked to embryo blastocyst rates (Houghton, 2002), human embryo live
104 birth rates (Brison *et al.*, 2004), DNA damage (Sturmey, 2009), aneuploidy (Picton *et al.*,
105 2010) embryo sex (Sturmey *et al.*, 2009a), maternal age (Picton *et al.*, 2010) and
106 embryonic stress (Wale and Gardner, 2012).

107

108 When considering energy metabolism of early embryos, it is vital that the contribution
109 made by endogenous triglyceride is not overlooked. Fatty acid β -oxidation was studied in

110 detail in the 1970s by Kane and colleagues (1979) but then largely ignored, with the
111 notable exception of the work by Downs (see Downs 2015). However, interest in fatty acid
112 metabolism has re-awakened, partly in response to the report from Dunning *et al.*, (2010)
113 who elegantly demonstrated that mouse oocytes require fatty acid oxidation in order to
114 develop. A similar conclusion was drawn by Sturmey and Leese (2003) in the pig,
115 underlining the importance of fatty acid β -oxidation during oocyte maturation, development
116 and in the preimplantation stages. Species differences in the importance of fatty acid
117 oxidation during oocyte and embryo development have also been identified. For example,
118 where a mouse zygote will arrest after 15 hours in media lacking nutrients (cited in Leese,
119 2012) a rabbit embryo can complete up to 3 cleavage divisions in the absence of energy
120 substrates (Kane, 1987) and sheep embryos can also develop to the blastocyst stage in
121 the absence of glucose (Thompson *et al.*, 1992). This can be explained by the differences
122 in intracellular triglyceride content, acting in a buffering capacity by providing an alternate
123 energy source (Ferguson and Leese, 2006; Sturmey *et al.*, 2009). Recently, a number of
124 laboratories have described altered fatty acid metabolism by embryos from overweight and
125 obese mice (Pantasri *et al.*, 2015; Reynolds *et al.*, 2015) and the human (Leary *et al.*,
126 2014). After receiving comparatively little attention since the work of Kane, interest in fatty
127 acid metabolism by oocytes and embryos has been intense, and has been widely
128 reviewed in recent years (Downs, 2015, Dunning *et al.*, 2014, Leroy *et al.*, 2012,
129 McKeegan and Sturmey, 2012, Sturmey *et al.*, 2009).

130

131 This very brief overview is intended to remind the reader of the basic features of early
132 embryo energy metabolism. However, 'metabolism' refers to significantly more functions
133 than ATP generation. For example, there is an extensive literature describing the role of
134 the pentose phosphate pathway (Downs *et al.*, 1998; Sutton-McDowall *et al.*, 2010) in

135 mammalian oocytes and early embryos. Moreover, metabolic processes link to signaling
136 mechanisms (Manser and Houghton, 2006), generation of Reactive Oxygen Species
137 (Agarwal *et al.*, 2005) and gene expression in terms of establishment of epigenetic marks
138 such as methylation and acetylation and post-translational modifications of proteins
139 (DeBerardinis and Thompson, 2012). For example, defects in folate metabolism have
140 been linked to methylation and epigenetic modifications affecting developmental
141 competence (Xu and Sinclair, 2015). However, reviewing all of the literature on embryo
142 metabolism in it's broadest sense would require several articles and so in the remainder of
143 this article, we will consider some more fundamental aspects.

144

145 **Why do we study embryo metabolism?**

146 Understanding the basic physiology and metabolism of the early embryo is a noble quest
147 in itself that has fascinated researchers over the past decades. However, a major gap in
148 our knowledge is the metabolism of the *in vivo* produced embryo, as well as the embryo *in*
149 *situ*, which remain an elusive goal. We aim to gain information that can, and has been,
150 translated into clinical practice in many ways; to design appropriate species specific
151 culture media with the aim of producing viable healthy offspring; to design non-invasive
152 methods for embryo selection for transfer and shed light on metabolic perturbations
153 occurring *in vivo*. Moreover, as our understanding of somatic cell nuclear transfer (SCNT;
154 Wilmut *et al.*, 2002) grows and becomes linked inextricably to stem cell physiology and
155 regenerative medicine, we must also accept that we know comparatively little about the
156 impact of such techniques may have on embryo physiology. Furthermore, we are on the
157 brink of many new and exciting developments in Assisted Conception, including
158 mitochondrial transfer for the treatment of debilitating hereditary conditions as well as the
159 replenishment of mitochondria in aged oocytes with the aim of improving pregnancy rates

160 in older women (Craven *et al.*, 2010; Smeets, 2013). Such techniques may be considered
161 'beyond experimental'; mitochondrial transfer was licensed for treatment in the UK in 2014
162 and autologous mitochondrial transfer for infertility is already commercially available in
163 some countries. However, since each of the approaches described above involve, in
164 some way, altering the mitochondrial content of embryos, the need for detailed
165 understanding of metabolic regulation of individual preimplantation mammalian embryo
166 has never been greater.

167

168 A further drive to study embryo metabolism comes from the need to identify biomarkers of
169 embryo health and viability. This relies on the inherent variability in metabolism between
170 different embryos and has been used in an attempt to select viable embryos for transfer,
171 with the end goal being clinical IVF in humans. There have been several observations that
172 have yielded promising results. The 'quiet embryo hypothesis' proposed by Leese in 2002,
173 stated that those embryos that are viable have a decreased metabolic rate; a proposition
174 that has been supported by several studies showing embryos with an upregulated
175 metabolism of both carbohydrates and amino acids to have decreased viability post
176 transfer (Guerif *et al.*, 2013, Lane and Gardner, 1996, Sturmey *et al.*, 2009). However, the
177 notion is contested, and there are recent studies suggesting that elevated metabolism,
178 particularly with respect to glucose consumption is associated with embryo viability
179 (Gardner *et al.*, 2011). Clearly, this is an area in which more work is needed.

180

181 Since pioneering observations linking human birth weight to cardiovascular events in later
182 life by David Barker (1989) it has now been shown unequivocally in many species that the
183 periconceptual environment can have downstream effects which can impact on the viability
184 of the developing embryo and on the future health of the resulting offspring (Ceelen *et al.*,

185 2008, Fleming *et al.*, 2012, Frank *et al.*, 2014, Leroy *et al.*, 2009, Watkins *et al.*, 2008). It is
186 also clear that certain embryonic stages are more susceptible to damage (Rieger, 1992),
187 such as the early cleavage embryo during embryonic genome activation, suggesting that
188 progeny may have a 'memory' of their origins.

189

190 With the rising obesity epidemic both in humans and companion animals, in addition to
191 metabolic disease in farm animal species due to increased production pressures, the study
192 of embryo metabolism *in vitro* can provide insight into the mechanisms of resultant
193 suppressed fertility and potentially identify therapeutic interventions.

194

195 These are important reasons for studying embryo metabolism, and it is clear that
196 metabolic processes can directly influence gene expression (Van Hoesck *et al.*, 2013, Van
197 Hoesck *et al.*, 2011), and patterning of the embryo (Leary *et al.*, 2014). However, it is also
198 of fundamental importance to be aware of what is measured when studying embryo
199 metabolism. In the final part of this review, we will describe the strengths and limitations of
200 embryo metabolic studies.

201

202 **What are we actually measuring?**

203 The measurement of embryo metabolism is faced with many technical challenges.
204 Critically, the *in vivo* environment is still largely unknown for most species, meaning that
205 the extrapolation of knowledge to an embryo *in vivo* is of questionable validity. The data
206 available on embryo metabolism inform us of the strategy of substrate depletion and
207 appearance in a given milieu. *In vitro*, this milieu is constrained by the addition of a limited
208 number of substrates at static levels; supply and ratio of substrates varies only in response
209 to an embryo's own activity. This is in stark contrast to the situation *in vivo*, which is

210 dynamic and responsive (Leese *et al.*, 2008). Even in species for which the *in vivo*
211 embryo environment has been described, the method used to define it should be noted.
212 Often *post mortem* changes and/or inflammatory changes due to catheterization can
213 influence results thus making samples non-representative (Leese *et al.*, 2008). Moreover,
214 the embryo *in situ* likely exists in a microenvironment within the oviduct, thus any subtle,
215 specific composition features will be lost in flushing of the tube.

216

217 Given the heterogeneity in developmental potential, measures pertaining to single
218 embryos are key and thus highly sensitive assays are needed. Both the use of
219 radiolabelled substrates (Rieger *et al.*, 1992) and enzyme-linked fluorescence assays to
220 detect the appearance and disappearance of a substrate from culture media have been
221 described (Leese and Barton, 1984; Guerif *et al.*, 2013). The relative metabolic quiescence
222 of single embryos means that ‘analysis media’ (that is a medium in which the
223 concentrations of substrates is reduced to enable measurement of change) is often used in
224 order to permit detection of changes in substrate concentration (Hardy *et al.*, 1989;
225 Sturmey and Leese, 2003). This ‘analysis medium’ is often different to the *in vitro* culture
226 media known to support development for most species, which, in turn differs vastly to the
227 *in vivo* environment. Of course, it also must be realized that there are many complex cell
228 transport and metabolic pathways involved, and notions of influx and efflux leads’ us to
229 make what are essentially educated guesses about what occurs in the cell. Despite these
230 limitations, these assays have greatly advanced our knowledge of metabolic pathways
231 involved and have yielded highly repeatable results across different laboratories. Further
232 methods that have been used to detect metabolic activity of embryos include culturing
233 individually in micro-droplets or in large groups of embryos. However, the resolution of

234 data from group culture is reduced since individual embryo heterogeneity is lost by
235 'averaging'.

236

237 New promising studies using NMR metabolomic technology, where substrate flux can be
238 measured in situ have been recently described (Krisher *et al.*, 2015), however the
239 subsequent interpretation and analysis of the complex data acquired presents new
240 challenges.

241

242 Inferences about the contribution of oxidative metabolism are usually derived from
243 measuring oxygen consumption. Methods vary, the most widely used being pyrene
244 fluorescence (Houghton *et al.*, 1996) and nanorespirometry (Lopes *et al.*, 2010). Again
245 while allowing accurate measurement of oxygen depletion in single embryos and
246 seemingly not affecting development (Lopes *et al.*, 2005), the methods represents a
247 significant 'alien' environment for the embryo.

248

249 Studies involving metabolic inhibitors and enzymatic co-factors have also added to our
250 knowledge of embryo metabolism and in some cases provided the initial proof of certain
251 pathways occurring and either being essential or non-essential for development. Among
252 these, Brison and Leese (1994) showed that oxidative phosphorylation was not an
253 absolute requirement for blastocoele formation in the rat by culturing embryos in the
254 presence of cyanide, while Macháty and colleagues (2001) indicated that suppression of
255 oxidative phosphorylation at the morula stage improved development to the blastocyst in
256 the pig. Moreover, Dunning *et al.*, (2010) have shown that β -oxidation is essential for
257 optimal development in the mouse by culturing in the presence of etomoxir. In some
258 cases, inhibition of certain metabolic pathways has been shown to improve developmental

259 potential; for example the addition of EDTA to embryo culture medium (Gardner *et al.*,
260 2000). Although the mechanism is not confirmed, one possible role of EDTA in embryo
261 culture medium is the suppression of glycolysis (Gardner *et al.*, 2000). However, it is
262 equally likely that EDTA acts as an antioxidant by sequestration of metal ions which
263 would otherwise catalyse the formation of Reactive Oxygen Species (Orsi and Leese,
264 2001). Studies such as these illustrate the importance of appropriate regulation of
265 metabolic pathways during development and also indicate why it is necessary for
266 pathways to be correctly orchestrated to match needs at a given stage of development.

267

268 **It all depends on the environment**

269 It could be argued that measuring embryo metabolism *in vitro* (by necessity) amounts to
270 measuring a stress response. This issue must be considered given the extremely
271 adaptable nature of embryos of all species. Metabolism is necessarily dynamic, enabling
272 rapid changes in needs to be met to maintain development. However, such dynamism
273 means that the metabolic profile of an embryo can respond quickly in response to a
274 change in external environment, shown clearly in mice, where perturbations occur within 3
275 hours of *in vitro* culture in flushed *in vivo* blastocysts (Lane and Gardner, 1998). Both the
276 presence and relative quantities of metabolic substrates in the environment in which
277 experiments are conducted will significantly affect the results. While not attempting to
278 provide a detailed discussion on the controversial aspects of *in vitro* culture systems,
279 which still vary widely across laboratories, this point can be further illustrated by the
280 differential metabolism that results from the presence or absence of serum and the
281 atmospheric oxygen concentration (Wale and Gardner 2010).

282

283 While the human IVF industry has moved towards defined culture media using
284 macromolecular sources such as recombinant albumin, serum is still used in many
285 production animal systems. Culture with serum has been shown to increase blastocyst
286 development rates in the horse (Choi *et al.*, 2004) and the kinetics of blastocyst
287 development in the cow (Rizos *et al.*, 2003). However, its presence has also been
288 associated with increased intracellular lipid content (Ferguson and Leese, 2006) and
289 altered metabolism (Reis *et al.*, 2003), up-regulation of oxidative stress and inflammatory
290 pathways (Cagnone & Sirard, 2014) and decreased survival after vitrification (Gómez *et*
291 *al.*, 2008). In addition, the oxygen tension of the reproductive tract in all species studied
292 has been found to be below 10% (Fischer and Bavister, 1993), In terms of the
293 environmental gas profile, there is now unequivocal evidence to support the notion that
294 20% oxygen reduces embryo development (Thompson *et al.*, 1990, Wale and Gardner,
295 2010) and that culture in low oxygen (5%) results in metabolic and proteomic profiles more
296 closely matching *in vivo* counterparts (Thomson *et al.*, 1990; Katz-Jaffe *et al.*, 2005).
297 Clearly, these factors will influence the results of any metabolic study and must be kept in
298 mind when comparing studies.

299

300 In addition to the embryo adapting to its environment, the culture environment itself is not
301 static. Depletion and accumulation of excreted of substrates such as lactate and amino
302 acids will change the local environment. Spontaneous de-amination will occur at 37°C,
303 especially of glutamine, resulting in ammonium build up (Gardner and Lane, 1993), lactate
304 build up may overwhelm pH buffering system of the media and depletion of energy
305 substrates can lead to alternative ATP generating pathways being used (Kane, 1987).

306

307 It is also important to note that the manner in which an embryo responds to its
308 environment is species specific. This can be seen in differences in response to
309 hyperglycaemia. While species such as rodents and humans, will have significant
310 diminished development in the presence of high glucose (Moley *et al.*, 1998; Frank *et al.*,
311 2014), others such as the horse and pig are apparently unaffected (Choi *et al.*, 2015,
312 Sturmey and Leese, 2003). Qualitative testing of equine embryos produced in
313 hyperglycaemic conditions however, highlights subtle differences not reflected in the
314 blastocyst development rate such as a decrease in ICM cell number allocation (also
315 observed in the rat) and known to be mediated through apoptosis (Moley *et al.*, 1998; Choi
316 *et al.*, 2015).

317

318 It is thus vital to consider that studies on embryo metabolism provide us a snapshot of
319 physiology *in a given set of conditions*. Whilst such data are of fundamental importance,
320 care must be taken when extrapolating and comparing such information. It is thus much
321 more desirable that studies on the depletion and appearance of embryo metabolism are
322 reinforced by consideration of mechanisms of metabolic regulation of early development.

323

324 **Embryo metabolism: some unanswered questions**

325 As the emphasis in human IVF is increasingly on single embryo transfer, the identification
326 of reliable non-invasive methods of determining embryo quality to maximize pregnancy
327 rate per transfer remains the Holy Grail. Moreover, in species such as the horse where *in*
328 *vitro* embryo production is rapidly generating interest, a specific tailored culture media has
329 yet to be formulated. Whilst acceptable blastocyst rates (41%) and pregnancy rates after
330 transfer (66%) can be achieved by some laboratories in the horse using cell culture media
331 such as DMEM-F12, (Jacobson *et al.*, 2010; Hinrichs *et al.*, 2014) the more subtle effects

332 of potentially inappropriate culture conditions leading to decreased viability remain to be
333 seen. Identifying optimal species-specific culture systems presents an exciting challenge
334 for those involved in studying embryo metabolism.

335

336 Sex selection is another lively area of embryo metabolism. Ethical considerations preclude
337 the implementation of sex selection in the human, but in the production animal industry,
338 and in dairy cattle in particular, appropriate non-invasive identification of sex before
339 transfer would be an application with many uses. Promising results have been presented
340 so far showing that both glucose metabolism and amino acid metabolism varies with sex
341 (Sturmey *et al.*, 2010; for review see Gardner *et al.*, 2010), however more work will need
342 performed to increase specificity in order for the technology to make the transition to
343 commercial practice.

344

345 New information is emerging all the time on the far-reaching downstream effects of
346 aberrations in early embryo metabolism (Harrison and Langley-Evans, 2009). Given the
347 clear links between the periconceptual environment and sub-optimal health outcomes in
348 the human (Barker *et al.*, 2002) and production species such as the bovine (for example,
349 the so-called Large Offspring Syndrome; Young *et al.*, 1998), understanding and
350 attempting to mitigate the negative effects on suboptimal embryo development and life-
351 long health of the offspring is an important area for future study (Leese 2014).

352

353

354 **Conclusions**

355 It is acknowledged “that metabolism pervades every aspect of cell physiology”
356 (DeBerardinis and Thompson, 2012) and this is especially pertinent to the developmentally

357 plastic early mammalian embryo. As genomic, transcriptomic and imaging techniques
358 advance we will be able to expand our understanding of embryo metabolism and how it
359 links inextricably with developmental pathways through subsequent stages of gestation
360 leading to the birth of a healthy offspring. It is the responsibility of us all working in the
361 earliest stages of this process to understand the periconceptual environmental challenges
362 faced by the embryo and to optimize the conditions under which it is grown to ensure the
363 best start in life. Metabolic studies allow us to gain vital information on the requirements of
364 a competent embryo and identify when things go wrong, but the reader is cautioned
365 towards careful interpretation of measures of metabolism especially between laboratories
366 and to consider the environment as a whole under which they have been taken.

367

368 **Acknowledgements**

369 NL is funded by the UK BBSRC. The authors are grateful to Professor Henry Leese for
370 critical comments in the drafting of this article.

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